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13. ABSTRACT *(Maximum 200 Words)*

Transfection of normal mammary epithelial cells with ErbB2 induced tumorigenic transformation and was associated with increased c-src activity. Transformation with other oncogens did not affect c-src activity. Induction of c-src activity was found to be associated with decreased phosphorylation on Y527 of c-src. This decreased Y527 phosphorylation was associated with increased association of the phosphotyrosine phosphatase SHP2 with ErbB2. Dominant negative SHP2 inhibited association of phosphotyrosine phosphatases with ErbB2 and ErbB2-induced src activity. Additional studies indicated that while ErbB2-induced c-src activation was not required for cell growth, it appears to be an important factor in tumorigenic transformation. In particular, c-src activation appears to be required for soft agar growth and tumor formation in athymic mice. These results suggerst a model of ErrbB2-mediated tumorigenesis in which ErbB2 induces activation of SHP2, which activates c-src, leading to increased tumor formation.

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INTRODUCTION

The hypothesis of this proposal is that ErbB2 expression regulates the activity of c-src and that this activation is a factor in mediating ErbB2 induced tumorigenesis. Previous work with human tumors has shown increased c-src activity (Ottenhoff-Klaff et al., 1992). Tissue from mice overexpressing ErbB2 show increased activity of c-src (Muthuswamy et al., 1994). Knockout mice lacking c-src also exhibit decreased tumorigenesis induced by polyomavirus middle T antigen (Guy et al., 1994). Data from our laboratory and others (Zhair et al., 1993) show that non-tumorigenic mammary epithelial cells are readily transformed to a tumorigenic phenotype by overexpression of ErbB2. This tumorigenic transformation is accompanied by increased activity of c-src (see below).

As described in the STATEMENT OF WORK, the project consisted of:

I.   Verify ErbB2 overexpression activation of c-src and map phosphorylation sites of src.

II.   Determine if src activation by ErbB2 is correlated with altered activity of src kinases or phosphatases

III. Evaluate the effects of inhibiting c-src activity on ErbB2-mediated tumorigenesis.

BODY

Experimental Methods

Cell Lines

The nontumorigenic human mammary epithelial cell line 184.A1 was used in most studies covered by this report. For comparison, the nontumorigenic huyman line MCF10A and the nontumorigenic mouse line NMuMG were also used when indicated. Cells were routinely grown in DMEM:F12 + 10% FBS, 5 µg/ml insulin and 10 ng/ml EGF).

Cells were transfected by ErbB2 and with v-Ha-ras in vectors containing a G418 resistance marker. Vectors were cloned in DH5α E. coli using standard procedures. Cells (5×10^6 in 0.5 ml HBSS) were placed in electroporation cuvets with 2 mm electrode space and pulsed with 1.2 kV/cm field strength. Cells were left on ice for 10 minutes, then returned to culture media. After 24 hours, media was changed to contain 400 µg/ml G418 sulfate and selection continued for 4 weeks. Other transfections included dominant negative src (Bell et al., 1992) and dominant negative SHP2 (Servidei et al., 1998), using methods described above.

Characteristics of cell lines

ErbB2 expression

To assess overxpression of ErbB2, cells were plated in 60 mm culture dishes (10^7 cells per dish and grown for 24 hours. Media was removed and SDS loading buffer lacking mercaptoethanol and bromophenol blue added to plates. Cells were scraped into tubes and heated to 95°C for 5 minutes. Protein content was then assessed by BCA assay (Pierce Chemical Co., Rockford, IL) and equalized among samples. Bromophenol blue and 2-mercaptoethanol were added to samples, equal protein was then separated by SDS-PAGE using a 7.5 % separating gel (Laemmli, 1970) and transferred to PVDF membranes (Towbin et al., 1979; Fenton and Sheffield, 1993). Membranes were probed with anti-ErbB2 (Transduction Laboratories, Lexington, KY) and detected with chemiluminescence (DuPont, Boston, MA) as described previously for other proteins (Fenton and Sheffield, 1993). Band intensity was quantitated by computer assisted densitometry (Collage®, Fotodyne, New Berlin, WI).

Growth on soft agar

Cells (10^4) were suspended in 2 ml of 0.3% agarose dissolved in culture media and layered

onto 2 ml of hardened 0.5% agarose in culture media. After 10 days, cells were observed for

growth.

Growth in nude mice

Inguinal (fourth) mammae of 3 week old athymic mice were exposed and epithelial

containing portions excised. Cell suspension (10 μl of a suspension of 1×10^8 cells/ml in HBSS)

were injected into the resulting fat ape and incisions closed with wound clips. Mice were observed

daily for tumor formation and euthanized with 5 mg pentobarbital i.p. when tumors were palpable.

Tumors were processed for histological evaluation by fixing in phosphate buffered formalin,

embedding in plastic, cutting into 5 μm thick slices, mounting on slides and staining with

hematoxylin and eosin.

Measurement of c-src activity

Cells were lysed with lysis buffer (30 mM sodium pyrophosphate, 10 mM EDTA, 50 mM

NaCl, 50 mM NaF, 1% Triton X-100, 0.1% BSA, 1 mM sodium orthovanadate, 1 mM PMSF).

Lysates were clarified by centrifugation for 10 minutes at 15,000 g, protein content determined by

BCA assay and protein equalized among samples. Samples were then incubated for 2 hours at

room temperature with anti c-src and agarose conjugated protein A and G. Samples were

centrifuged and beads washed 4 times with lysis buffer. Beads were then incubated with kinase

buffer (10 μl of 200 mM HEPES, pH 7.0 containing 125 mM $MgCl_2$, 25 mM $MnCl_2$ and 0.25

mM sodium orthovanadium. Substrate (5 μl of a 0.5 mM solution of [lys^{19}]cdc2(6-20) was added

and reactions started by adding 5 μl of γ^{32}P-ATP (0.5 mM, specific activity of 5,000 dpm/pmole).

After 5 minutes, reactions were stopped by adding 10 μl of 50% acetic acid and samples were

centrifuged (5,000 g for 5 minutes). Supernatant was spotted onto Whattman P81

phosphocellulose paper, washed 4 times with cold 100 mM phosphoric acid, rinsed with acetone,

dried and counted by liquid scintillation. In addition, reactions were performed without peptide or

with [val^{12}ser^{14}lys^{19}]cdc2(6-20) (which should not be phosphorylated by src) as a substrate. c-src content of immunoprecipitates was determined by western blot analysis, essentially as described above for ErbB2 and resulting data used to correct for any differences in src content.

Measurement of c-src phosphorylation

C-src was immunoprecipitated as described above. Src was then digested with 50 mg/ml cyanogen bromide in formic acid and lyophylized. Resulting peptides were separated by tricine PAGE (van der Geer et al., 1993) and transferred to PVDF membranes by electroblotting. Membranes were then blocked and probed with anti-phosphotyrosine as previously described (Fenton and Sheffield, 1993). Density of bands was determined by densitometry (Collage®).

Measurement of c-src Expression

Cells were lysed as described above for ErbB2 expression. Protein content of lysates was equalized, proteins separated by SDS-PAGE (12% separating gel) and probed with anti-c-src as above.

Measurement of other src-related kinases

Other members of the src family, including lyk, lyn and fyn, were immunoprecipitated as described for c-src and activity estimated essentially as described for c-src.

Measurement of CSK content

Cells were lysed with SDS loading buffer and proteins separated by SDS-PAGE. Proteins were transferred to PVDF membranes, blocked with BSA and probed for CSK by Western blot analysis using chemiluminescence detection. Computer assisted densitometry was used to estimate relative band intensity.

Measurement of CSK activity

Cells were lysed with lysis buffer (50 mM HEPES containing 1 mM PMSF, 40 mM Sodium orthophosphate, 1 mg/ml BSA and 1% Triton X-100), clarified by centrifugation (14,000 g for 15 minutes) and CAK immunoprecipitated from lysates using a rabbit polyclonal antibody and agarose

conjugated protein A/G. Beads were washed with lysis buffer and then incubated with assay

buffer (200 mM HEPES containing 100 mM MgCl$_2$, 25 mM MnCl$_2$ and 0.1 mM Na$_3$VO$_4$) with

substrate peptide corresponding to the C-terminus of src (TSTEPQY(PO$_4$)QPENL). Assays were

begun by adding γ^{32}P-ATP (10 μM containing 250,000 dpm) and continued for 5 minutes.

Assays were stopped by adding 50% acetic acid and 1 mg/ml BSA. Tubes were then centrifuged

(14,000 g for 5 minutes) and supernatant spotted onto Whatman 3MM filter Paper. Filter paper

was washed four times with 100 mM phosphoric acid, dried and counted by liquid scintillation.

Background was estimated by performing the assay in the absence of substrate peptide and was

subtracted to give substrate-dependent activity.

Measurement of phosphatase activity

For assays on crude cell lysates, cells were lysed with 50 mM HEPES containing 40 mM

sodium pyrophosphate, 50 mM NaF, 0.1 mM Na^{3}VO^4 , 1 mM PMSF and 1% Triton X-100.

Lysate was concentrated with an Amicon centrifical concentrator and resuspended in phosphate-

free buffer. Protein content was determined by BCA method (Pierce Chemical Co., Rockford,

IL), equalized among samples and phosphatase activity assessed by the Malacite Green method of

assessing free phosphate (Harder et al., 1994) and using a peptide corresponding to the C-terminus

of src (peptide 301, Biomole, Plymouth Meeting, PA) or a peptide corresponding to the

autophosphorylation site of src (peptide 312, Biomole) as substrate peptides.

For membrane preparations, cells were lysed as described above except that the buffer lacked

Triton X-100, centrifuged (2000 g for 5 minutes) and supernatant centrifuged at 50,000 g for 60

minutes. Pellets were dissolved in phosphate free assay buffer containing 1% Triton X-100 and

phosphatase activity assessed as described above. Cytosol was assessed as described above for

cell lysates.

To assess phosphatase associated with ErbB2, ErbB2 was immunoprecipitated and resulting

immunoprecipitates assayed as described above.

Western Analysis of phosphatase content

Cell lysates, membranes or ErbB2 immunoprecipitates were prepared as described above.

Proteins were separated by SDS-PAGE, transferred to PVDF membranes and western blots

probed with antibodies to the indicated phosphatases. Computer densitometry was used to assess

relative band intensity.

Results

Characterization of cell lines

ErbB2 expression

Cells transfected with ErbB2 exhibited substantially greater concentrations of ErbB2 than

parental cells (Figure 1), indicating that the ErbB2 transfected lines dramatically overexpressed the

gene at the level of cell protein content.

ErbB2 Content of Cell lines
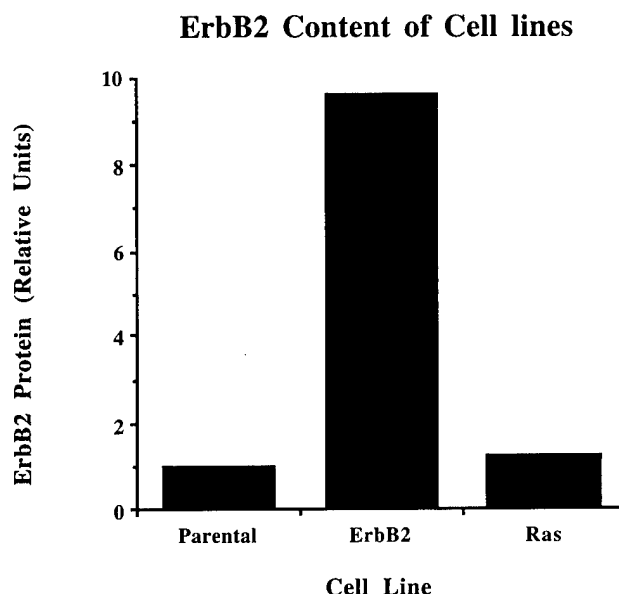
Figure 1. ErbB2 content of parental 184.A1 cells and cells transformed with ErbB2 and v-Ha-ras. Mean of 3 determinations.

Growth on soft agar

ErbB2 and ras transformed cells grew readily on soft agar, whereas the parental cells were

incapable of growing on soft agar. This, together with nude mouse tumor formation (below)

suggests that the cells are tumorigenically transformed.

Growth in nude mice

ErbB2 and ras transformed cells were capable of forming tumors in nude mice (100% of

inoculated mice had palpable tumors within 30 days). The tumors were typical of poorly

differentiated breast adenocarcinomas. No tumors were formed by the parental cells, suggesting

that ErbB2 and ras induced tumorigenic transformation.

c-src activity

Src activity (Figure 2) was dramatically increased by ErbB2 transformation, but only

modestly increased by ras transformation. When corrected for amount of c-src in

immunoprecipitates, essentially identical results were obtained, as c-src content of

immunoprecipitates exhibited little difference among treatments (Figure 3). This would be

consistent with a model in which ErbB2 induces c-src activity by activating existing enzyme, rather

than inducing c-src expression.
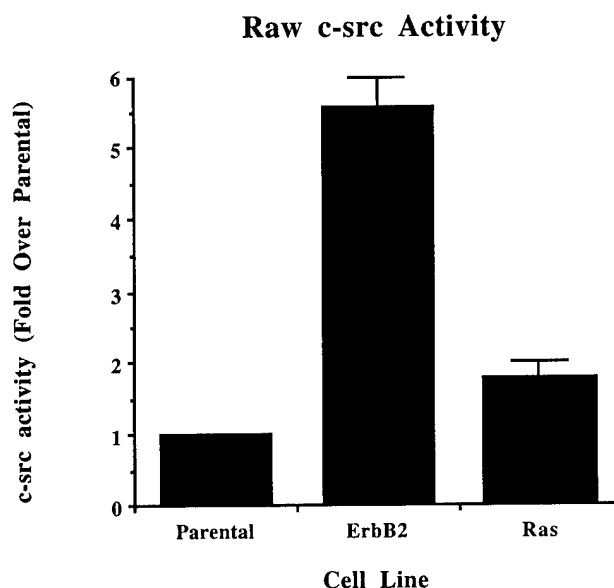
Raw c-src Activity

Figure 2. Effect of transformation by ErbB2 or Ras on total c-src activity in 184.A cells. Mean ±
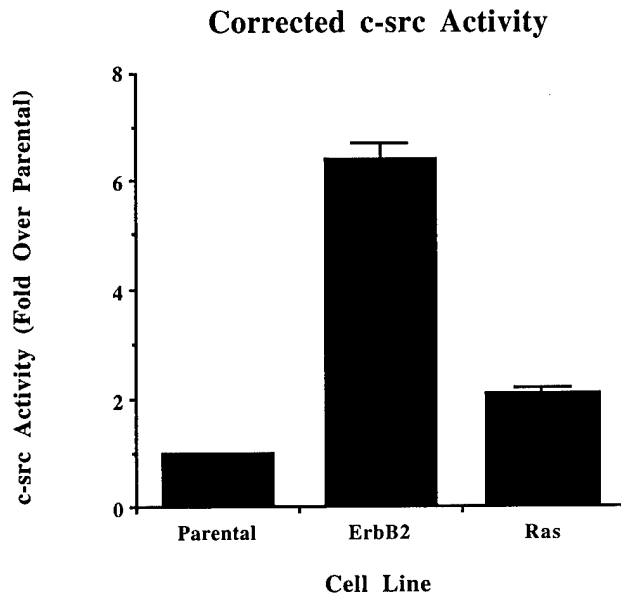SEM of 4 determinations.

Corrected c-src Activity

Figure 3. c-src activity in 184.A1 cells or 184.A1 cells transfected with ErbB2 or ras. Values reported after division by relative amount of c-src in the immunoprecipitate in order to give a specific activity change. Mean ± SEM of 4 observations.

Linearity of assay over time was verified by conducting assays for 2, 4, 6 or 8 minutes

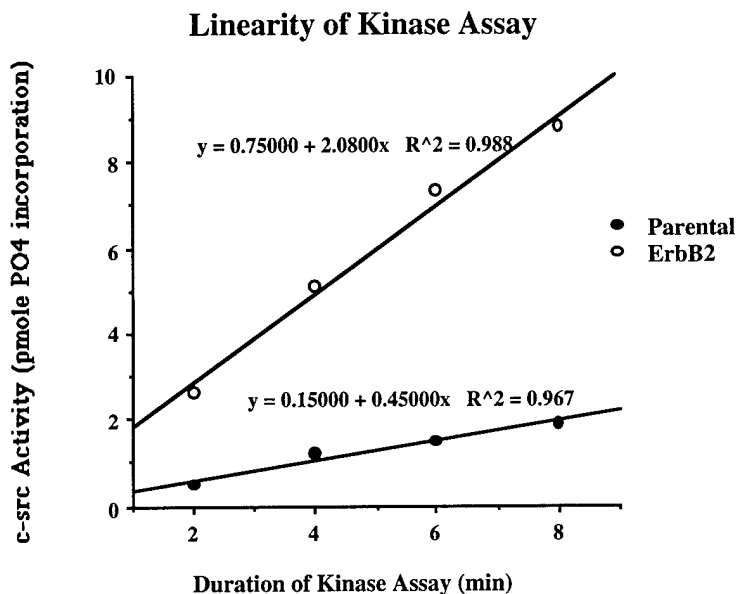(Figure 4) Results indicated that assay was linear over time.

Linearity of Kinase Assay

Figure 4. Linearity over assay time of c-src kinase assay on parental and ErbB2 transformed 184.A1 cells.

The c-src kinase assay used above was found to be dependent of added substrate peptide (Figure 5). Omission of the peptide or use of a peptide not phosphorylated by c-src yielded essentially background activity, as did omission of the enzyme.
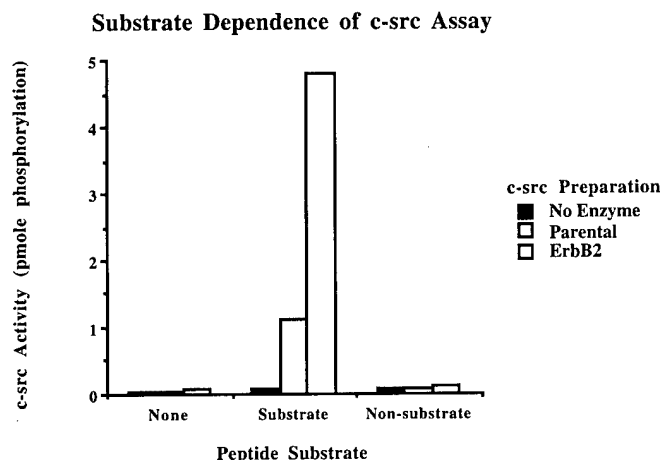
Substrate Dependence of c-src Assay

Figure 5. Effect of omission of enzyme, omission of substrate or substitution of a non-substrate peptide ([val^{12}ser^{14}lys^{19}]cdc2(6-20)) on c-src activity. Mean of 2 determinations.

Diluting the enzyme preparation used in kinase assays (Figure 6) caused a corresponding decrease in kinase activity, indicating that the results were linear over the enzyme concentration range used in the studies.

Linearity over Enzyme Concentration
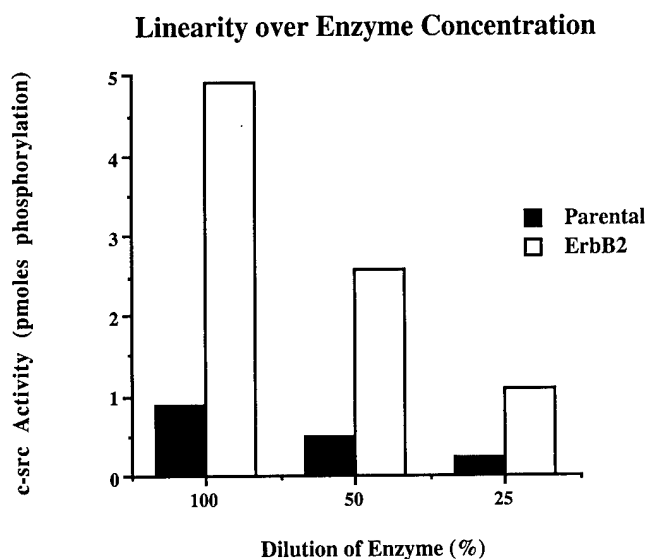
Figure 6. Effect of diluting enzyme preparation (immunoprecipitate) on measured c-src kinase activity. Mean of 2 experiments.

Performing the assay using different starting concentrations of substrate peptide indicated that

the results obtained were not an artifact of substrate concentration (Figure 7).
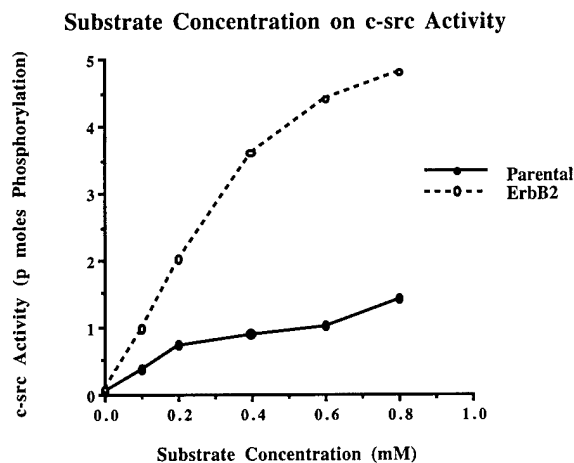
Substrate Concentration on c-src Activity

Figure 7. Dilution of substrate peptide on c-src kinase activity estimates in 184.A1 cells and cells
transformed with ErbB2. Mean of 2 determinations.

ATP concentration curve (Figure 8) indicated that kinase assays were conducted at near-

maximum concentrations of ATP, and that ATP depletion is not likely to be a factor in the results

obtained.

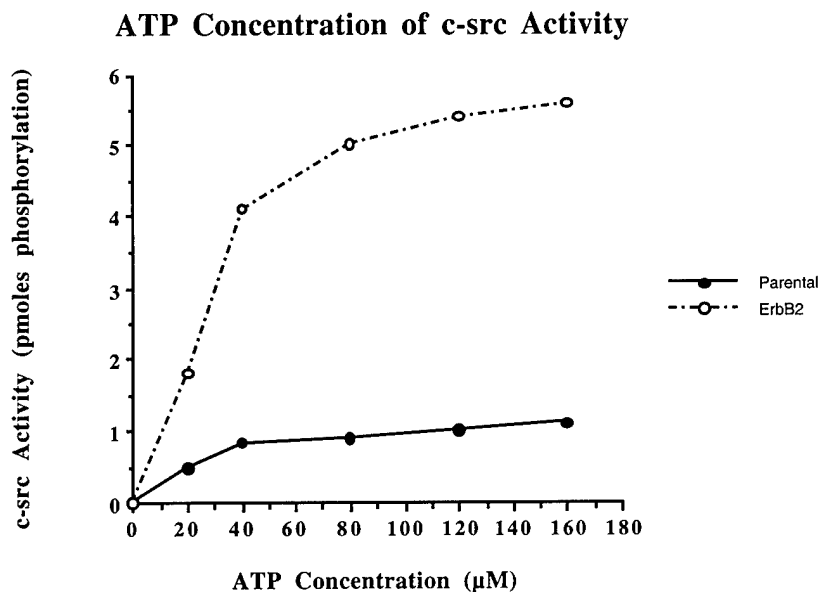**ATP Concentration of c-src Activity**

Figure 8. Effect of varying ATP concentration on c-src kinase activity estimates in 184.A1 cells
and 184.A1 cells transformed with ErbB2. Mean of 2 determinations.

c-src phosphorylation

Peptide mapping indicated a readily-detectable (approximately 3-4 fold) increase in tyrosine

phosphorylation of a 10 kDa cyanogen bromide peptide, corresponding to the autophosphorylation

site of c-src, and a corresponding decrease in tyrosine phosphorylation of a 4 kDa peptide (Figure

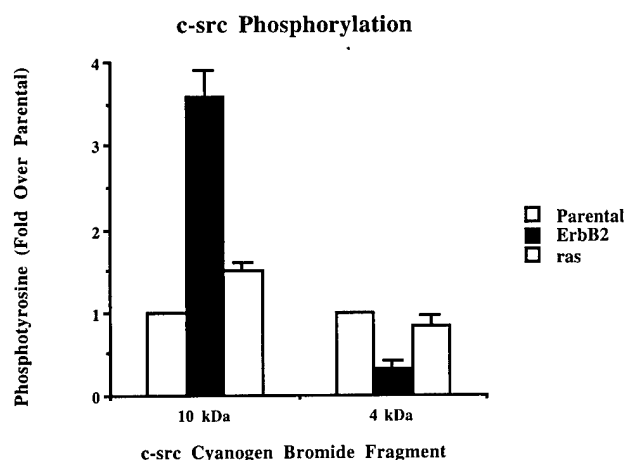9), which corresponds to the inhibitory tyrosine at position 527 (Nada et al., 1993).

Figure 9. Phosphorylation site of c-src in vivo in 184.A1 cells and cells transfected with ErbB2 or ras. Mean ± SEM of 3 determinations.

c-src Expression

Total c-src content of 184.A1 cells, ErbB2 transformed cells and ras transformed cells was

similar, indicating that changes observed above were likely to be due to activation of existing c-src,

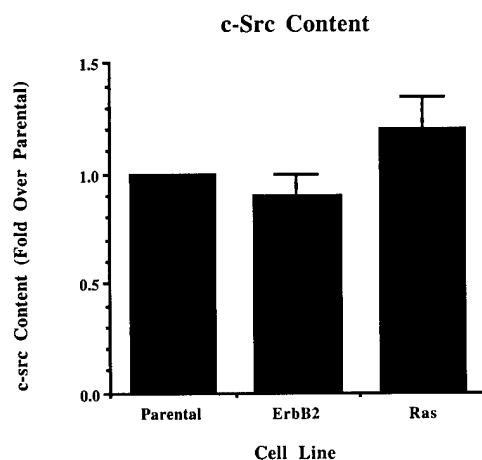not increased c-src expression (Figure 10).

Figure 10. Effect of ErbB2 or ras transformation of 184.A1 cells on c-src content, as determined by Western blot analysis. Mean ± SEM of 4 determinations.

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Other src-related kinases

To date, we have not observed activation of lyk, lyn or fyn in response to ErbB2

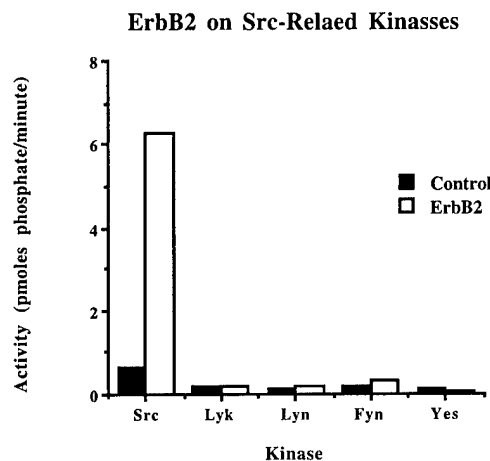transformation (Figure 11).

ErbB2 on Src-Relaed Kinasses

Figure 11. Activity of src-related kinases in vector-transfected (Control) and ErbB2-transformed 184.1 cells. Kinase was immunoprecipitated from indicated cells and activity determined as described in Methods. Mean of 3 determinations.

Other Cell Lines

Most of the work to date has been on 184.A1 cells and their transformed derivatives.

However, we have also transformed other cell lines with ErbB2 (some in preliminary studies

described in the grant proposal). These include NMuMG (a nontumorigenic mouse mammary

epithelial line) and MCF 10A (a non-tumorigenic human mammary epithelial cell line). In both of

these lines, ErbB2 increases c-src activity with little or no change in c-src protein content of cells.

Although baseline levels of c-src activity vary somewhat among cell lines, the fold induction by

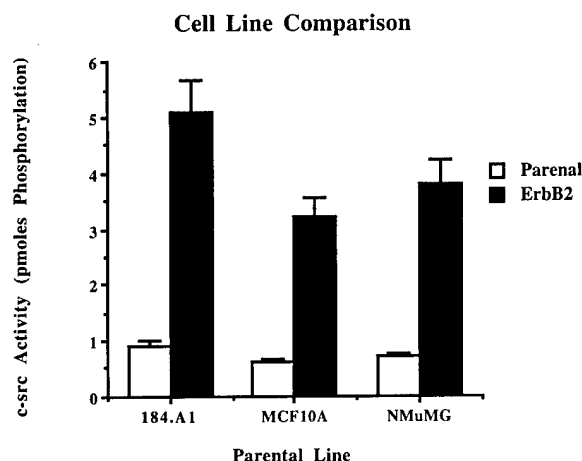ErbB2 was similar among lines (Figure 12).

Cell Line Comparison

Figure 12. Activation of c-src by ErbB2 transformation in various cell lines. Mean ± SEM of 3 determinations.

Expression and Activity of CSK

Since a possible mechanism by which ErbB2 could increase c-src activity is to decrease expression or activity of C-terminal Src Kinase (CSK), we examined the expression and activity of CSK in ErbB2 and Ras transformed cell lines. Ras had no significant effect on CSK level, whereas ErbB2 significantly increased CSK expression level (Figure 13). These effects were paralleled by a similar increase in CSK activity. When corrected for differences in CSK content, there was no effect of ErbB2 on specific activity of CSK. Thus, it appears that ErbB2 does not activate c-src by decreasing the rate at which Y527 is phosphorylated.
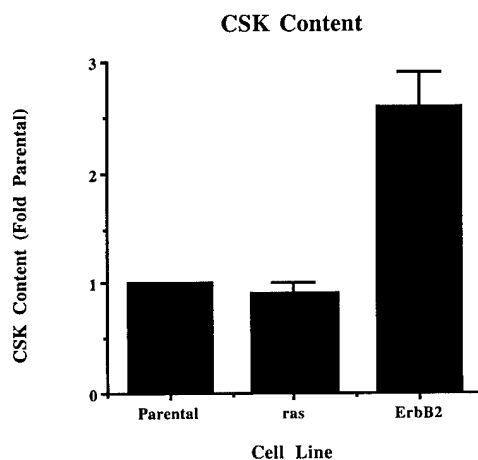
CSK Content

Figure 13. Effect of ErbB2 on CSK expression in 184.1 cells transformed with ErbB2 or ras.
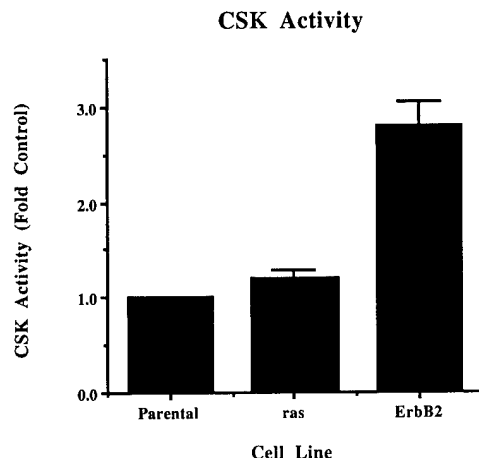
CSK Activity

Figure 14. Activity of CSK in 184.1 cells transformed with ErbB2 or ras.

Total Phosphatase Activity

To assess total phosphatase activity in various cell lines, two substrate peptides were used: 312, corresponding to Y416, the autophosphorylation site of c-src, and 301, corresponding to Y527, the CSK phosphorylated site of c-src. Substantially higher activity toward peptide 301 was observed, but there was no difference among cell lines in this activity. Lower activity was observed toward peptide 312, but this activity was slightly higher in ErbB2 transformed cell lines than in other lines.
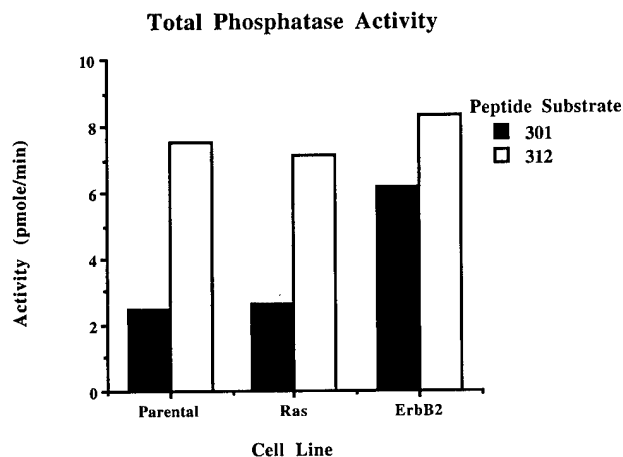
Total Phosphatase Activity

Figure 15. Effect of ErbB2 and ras transformation on total phosphatase activity of 184.1 cells toward c-src autophosphorylation site and CSK phosphorylation site.

Membrane associated Phosphatase Activity

Cell membranes and cytosol prepared from control, ras or ErbB2 transformed cells were assessed for phosphatase activity toward peptide 301 and 312. There was no difference among cell lines in peptide 312 dephosphorylation in either membrane or cytosolic fractions (Figure 16). In contrast, the membrane fraction of ErbB2 transformed cells exhibited substantially greater activity toward peptide 301 than other cell lines (Figure 17). However, the cytosolic fraction showed little difference in activity among the cell lines. These results indicated an increased membrane associated phosphatase activity with activity selective for Y527 of c-src. Further examination of the activity indicated that it was linear over the amount of membrane protein used (Figure 18) and over time (Figure 19). In addition, the activity was dependent on substrate, and near Vmax conditions were used in the standard assay (Figure 20).
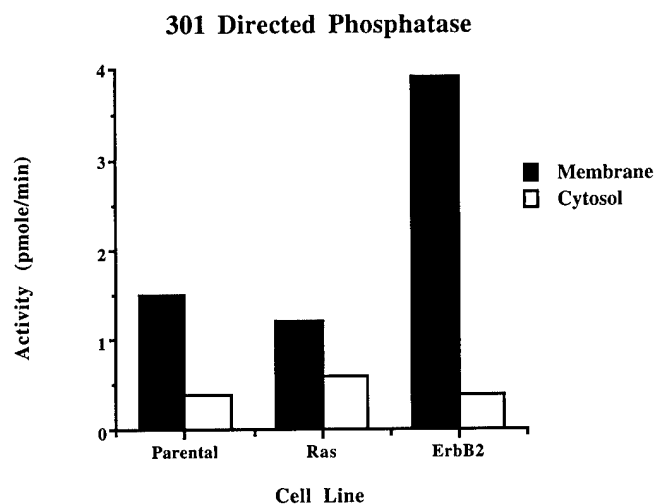
301 Directed Phosphatase

Figure 16. Effect of ErbB2 and ras transformation on activity of phosphatase toward CSK phosphorylation site in cell membranes and cytosolic fractions.
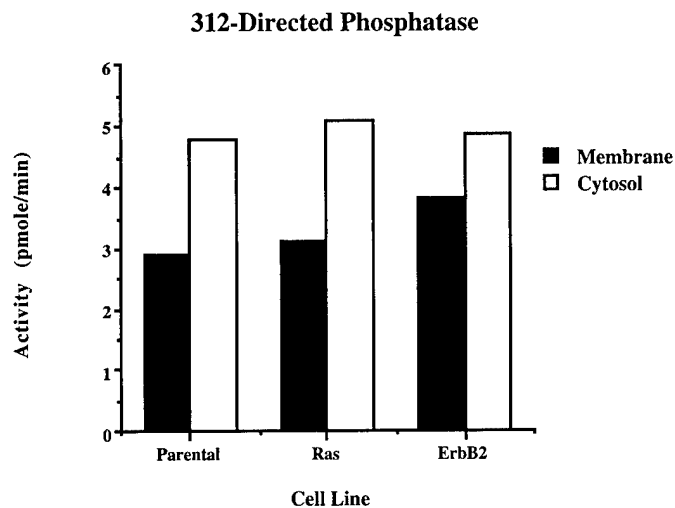
312-Directed Phosphatase

Figure 17. Effect of ErbB2 and ras transformation on activity of phosphatase toward c-src autophosphorylation site in cell membranes and cytosolic fractions.
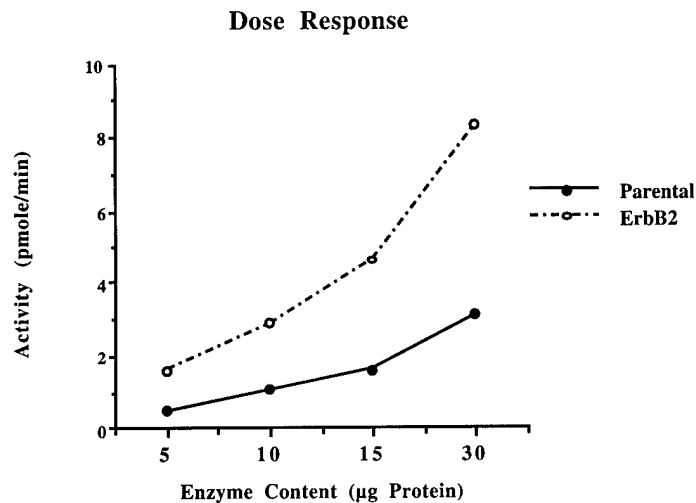
Dose Response

Figure 18. Linearity of phosphatase activity toward peptide 301 (CSK phoshorylation site of src) over amount of membrane protein assayed in Parental and ErbB2 transformed human mammary epithelial cells.
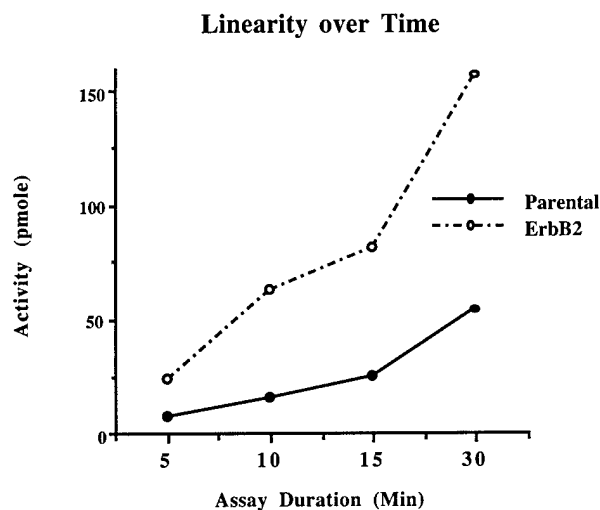
Linearity over Time

Figure 19. Linearity of phosphatase activity toward peptide 301 (CSK phoshorylation site of src) over time in Parental and ErbB2 transformed human mammary epithelial cells.
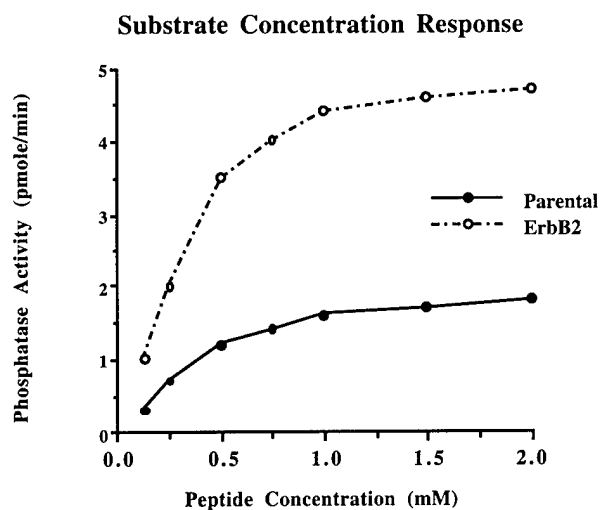
Substrate Concentration Response

Figure 20. Substrate kinetics of phosphatase activity toward peptide 301 (CSK phoshorylation site of src) over amount of membrane protein assayed in Parental and ErbB2 transformed human mammary epithelial cells.

ErbB2 associated phosphatase activity

Phosphatase activity in ErbB2 immunoprecipitates was assessed essentially as for membrane

fractions. These studies indicated that ErbB2 was physically associated with a phosphatase with

selectivity toward Y527 of CSK, and that the amount of this enzyme that co-precipitated with

ErbB2 was dramatically increased (about 9 fold) in cells transfected with ErbB2 (Figure 21).

However, it should be noted that ErbB2 transformed cells express 9-10 fold more ErbB2, so that

the increased ErbB2 association may be due to the increased amount of ErbB2, not a specific

increase in phosphatase-ErbB2 association in transformed cells.

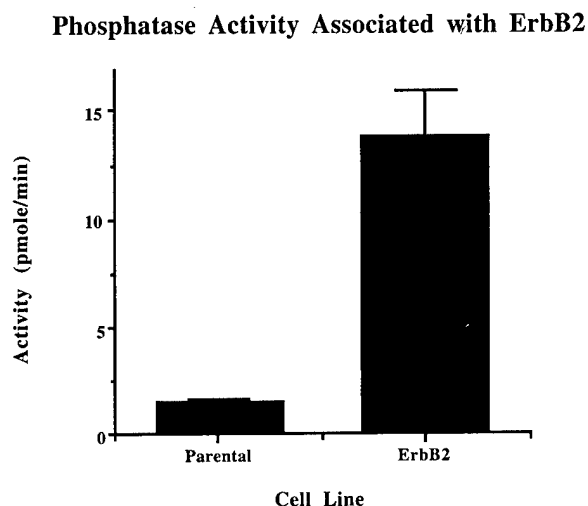**Phosphatase Activity Associated with ErbB2**

Figure 21. Phosphatase activity toward the CSK phosphorylation site of src in ErbB2 immunoprecipitates prepared from parental and ErbB2 transformed human mammary epithelium.

Specific Phosphatases

Western blot analysis indicated that the cell lines examined expressed detectable levels of PTP1B

and LAR, and these were increased by transformation. The cells expressed little SHP1 and SHP1

content was not affected by transformation. The cells expressed substantial amounts of SHP2, and

total SHP2 content was actually slightly lower in transformed cells (Figure 22). However, SHP2

associated with membrane fraction was substantially increased in ErbB2 transformed cells. In

addition, the amount of SHP2 present in ErbB2 immunoprecipitates was increased about 10 fold,

which is similar to the increase in ErbB2. These results, together with previous studies, suggest

that SHP2 associates with ErbB2. Transformation with ErbB2 does not dramatically alter the

specific association, but, due to greater ErbB2 expression, the total amount of SHP2 associated

with ErbB2 is dramatically increased. This provides a plausible mechanism for ErbB2 activation

of c-src, although that hypothesis has not yet been definitively tested.

**Phosphatase Association with ErbB2
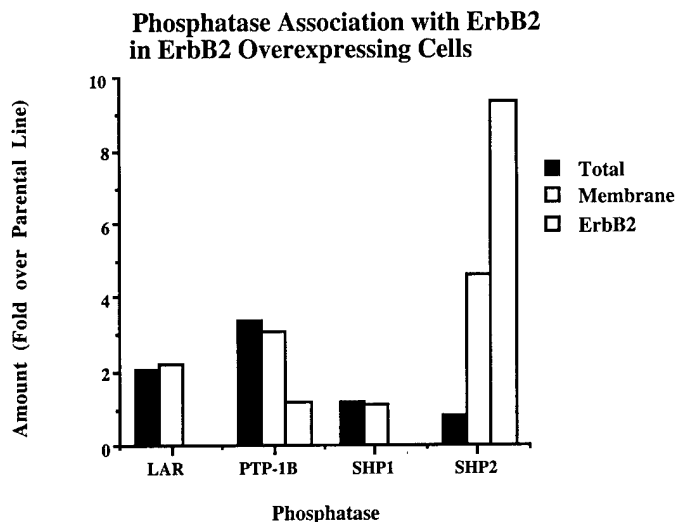in ErbB2 Overexpressing Cells**

Phosphatase

Figure 22. Expression level of specific phosphatases in ErbB2 transformed cell lines. Phosphatase content of crude cell lysates (Total), membrane preparations or ErbB2 immunoprecipitates were determined by Western analysis of parental and ErbB2 transformed human mammary epithelial cells and expressed as ErbB2 expression/Parental expression.

ErbB2-Dominant Negative c-src cell lines

In preliminary studies to characterize cell lines transfected with dominant negative c-src and ErbB2, the dominant negative mutant of c-src did not appear to inhibit cell proliferation on plastic. However, colony forming efficiency on soft agar was dramatically inhibited by dominant negative c-src when cells were transformed by ErbB2, but not by ras (Figure 23). Further evaluations of these cells are necessary, and scheduled for Year 3 of the project. These evaluations include replication of cell growth results, tumor formation in vitro, cell invasiveness assays and evaluations of c-src activity. However, the results to date indicate that c-src, although not necessary for cell proliferation, appears to be critical for ErbB2-mediated tumorigenic transformation.
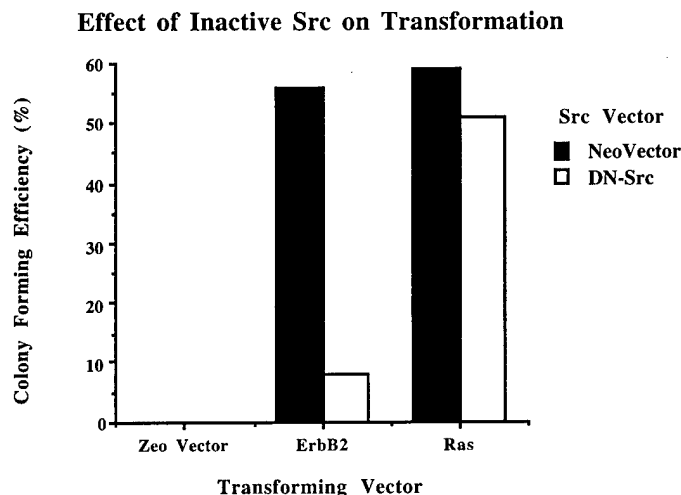
Effect of Inactive Src on Transformation

Figure 23. Colony forming efficiency of cells expressing combinations of ErbB2 and dominant negative src. Cells were transfected with a dominant negative kinase inactive src (DN Src) or vector alone (Neo Vector) and selected for G418 resistance. These cells were transfected with ErbB2, ras or vector alone (Zeo vector) and selected for Zeocin resistance. Cells were plated onto soft agar and colony forming efficiency deterined.

Association of ErbB2 with SHP2

Co-immunoprecipitation studies, followed by western blot analysis, indicated that a major

phosphatase associated with ErbB2 was SHP2 (Figure 24). SHP2 precipitated with ErbB2

appeared to largely parallel the amount of ErbB2 in samples, and the phosphatase activity directed

toward the c-terminus of c-src.

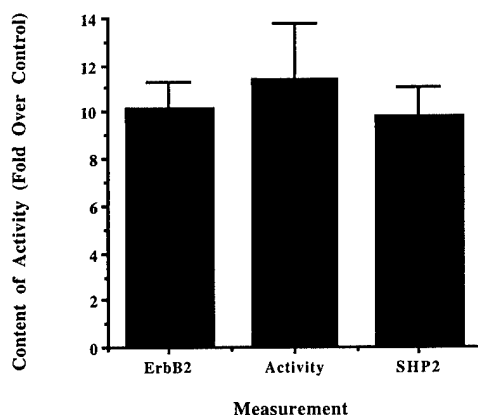Figure 24. Effect of ErbB2 on ErbB2-associated phosphatase. ErbB2 was immunoprecipitated from wild-type (Control) or ErbB2-transformed cells. ErbB2 and SHP2 content were determined by western blot analysis. Phosphatase activity was determined by de-phosphorylation of peptide corresponding to the C-terminus of c-src as described in Methods. Data expressed relative to wild-type (Control) cells. Mean ± SEM of 5 determinations.

Co-transfection of ErbB2 and dominant negative SHP2

To further assess the role of SHP2 in ErbB2-induced activation of c-src, cells were co-transfected with ErbB2 and vector alone (control) or ErbB2 and dominant negative SHP2 (a truncation mutation lacking the phosphatase domain of SHP2). Expression of dominant negative SHP2 dramatically reduced the association of phosphatase activity with ErbB2 (Figure 25), suggesting that a major phosphatase associated with ErbB2 is SHP2. In additional studies, the impact of dominant negative SHP2 on c-src activation by ErbB2 was assessed. Expression of inactive SHP2 prevented the ErbB2-induced activation of c-src (Figure 26). Thus, a major pathway for ErbB2-activation of c-src appears to be activation of SHP2.

SHP2 on ErbB2-Associated Phosphatase

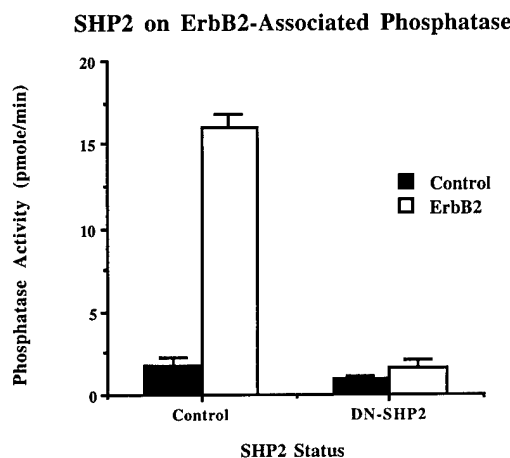Figure 25. Effect of dominant negative SHP2 on ErbB2-associated phosphatase activity. Cells transfected with vector alone (Control) or dominant negative SHP2 (DN-SHP2) were transfected with vector (Control) or ErbB2. ErbB2 was immunoprecipitated and phosphatase activity determined as dephosphorylation of peptide corresponding to the C-terminus of C-src. Mean ± SEM of 4 experiments.

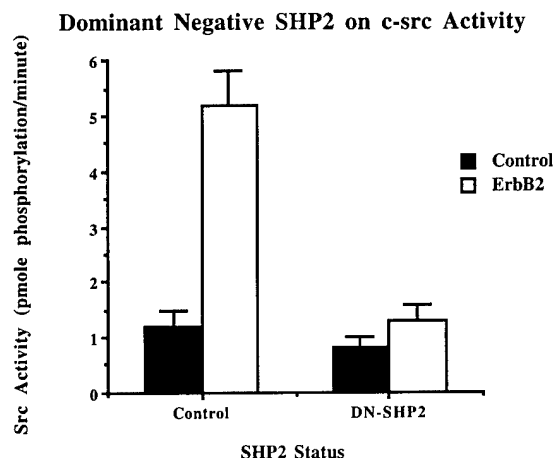**Page 25**

Dominant Negative SHP2 on c-src Activity

Figure 26. Effect of dominant negative SHP2 on ErbB2-induced c-src activity. Cells transfected with vector alone (Control) or dominant negative SHP2 (DN-SHP2) were transfected with vector (Control) or ErbB2. C-src activity was determined as described previously (Sheffield, 1998). Mean ± SEM of 4 experiments.

Src Phosphorylation in ErbB2 Immunoprecipitates
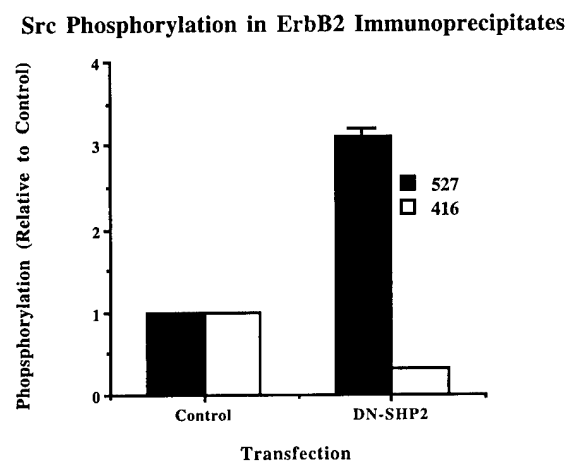
Figure 27. Effect of of SHP2 for src phosphorylation pattern. Cells co-transfected with vector (Control) or dominant-negative SHP2 and ErbB2 were used. ErbB2 was immunoprecipitated, co-p[recipitating src separated by PAGE and mapped by cyanogen bromide digestion as described in Methods. Mean ± SEM of 3 experiments.

Dominant negative src alters tumor formation

Previously, dominant negative c-src was found to inhibit the ability of mammary epithelial

cells to grow on soft agar. As an additional test of tumor formation, cells were xenografted to

athymic (nude) mice. After 2 months, 12/12 control tumors (ErbB2 and vector alone) were

detectable, whereas only 7/12 ErbB2-dominant negative src injections formed tumors.

Furthermore, the growth rate of the control cells was significantly faster, resulting in larger final

tumor weight (1.2 ± 0.3 g in controls vs 0.4 ± 0.2 g in dominant negative src expressing cells). These results indicate that c-src activation by ErbB2 appears to be necessary for tumor formation and development.

Discussion

Results of these studies indicate that ErbB2 does not dramatically alter the amount of c-src in cells, but does increase c-src activity 4-6 fold. This appears to be associated with increased in vivo tyrosine phosphorylation on the autophosphorylation site of c-src (tyrosine 416) and decreased tyrosine 527 phosphorylation. This would appear to represent a mechanism of activation of c-src (Cooper and Howell, 1993).

There are several possible mechanisms by which ErbB2 could activate c-src. ErbB2 could decrease expression or activity of the kinase responsible for phosphorylating tyrosine 527 (most likely csk or c-terminal src kinase, Cooper and Howell, 1993). Alternatively, ErbB2 could increase the activity or level of phosphotyrosine phosphatases directed toward the C-terminus of c-src. Results of these studies indicate that c-src activation is associated with decreased phosphorylation at Y527. Presumably, the decreased Y527 phosphorylation could be due to decreased CSK activity or increased phosphatase activity. The present studies indicate that decreased CSK activity does not appear to explain src activation by ErbB2. However, increased activity of SHP2 appears to be a plausible mechanism by which ErbB2 could activate src. In the present study, we determined that SHP2 was associated with ErbB2. This association does not appear to be modified by tumorigenic transformation, as the increase in SHP2 associated with ErbB2 immunoprecipitates was largely parallel with increased ErbB2 content of cells. Furthermore, dominant negative SHP2 reduced phosphatase activity associated with ErbB2 and dramatically incrased src Y527 phosphorylation. These results suggest that SHP2 or a SHP2-like phosphatase is likely to regulate ErbB2-induced src activity.

A second major objective of the project is to determine the consequences of c-src activation by ErbB2. These studies suggest that c-src activation by ErbB2 has little effect on growth rates, but is an important factor in tumor formation.

KEY RESEARCH ACCOMPLISHMENTS

-ErbB2 transformation of mammary epithelial cells leads to increased activity of c-src without increasing expression of c-src.

-C-src phosphorylation pattern in ErbB2 transformed cells was consistent with dephosphorylation at Y527 in response to ErbB2.

-c-src dephosphorylation on Y527 does not appear to be due to decreased CSK content or activity.

-c-src dephosphorylation in response to ErbB2 appears to be mediated by increased SHP2 activity.

-c-src activation by ErbB2 appears to be necessary for ErbB2-induced tumorigenicity.

REPORTABLE OUTCOMES

Manuscripts

Sheffield, L.G. 1998. Role of c-src activation in ErbB2-induced transformation of human breast epithelium. Biochem. Biophys. Res. Commun. 250:27-31.

Sheffield, L.G., Smuga-Otto, K., Lewandowski, J.A., Vilhubner, K. ErbB2 activates of c-src via the phosphotyrosine phosphatase SHP2. Manuscript in preparation.

Sheffield, L.G., Lewandowski, J.A. and Smuga-Otto, K. Requirements of c-src for ErbB2-induced STAT-mediaed transcription. Manuscript in preparation.

Sheffield, L.G. 2000. Involvement of SHP2 in ErbB2-mediated tumorigenesis. Era of Hope Meeting, 2000, Atlanta, GA.

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CONCLUSIONS

Results to date indicate that overexpression of ErbB2 in nontumorigenic mammary epithelial cells increases activity, but not expression, of c-src. Furthermore, this effect is not mimicked by other transforming oncogenes. These results suggest that previous results in transgenic animals are likely to be due to direct effects on mammary epitheliaum, not to alterations in systemic physiology (such as hormone levels) or to altered epithelial stromal interactions. The results also suggest a likely involvement of either csk or src-directed phosphatases in this activation. Examination of the mechanism of activation is the focus of ongoing work. These results suggest that strategies to modify src activation may be useful in the development of breast cancer therapies.

Previous results indicate that ErbB2 increases activity of c-src in mammary epithelium. The present studies suggest that this induction of c-src activity is mediated by increased activity of a src-directed kinase that removes an inhibitory phosphate at Y527. SHP2 appears to be a leading candidate for the phosphatase. In addition, the activation of c-src by ErbB2 appears to play a critical role in inducing a tumor phenotype. These results that strategies to modify src activity or the activity of the src-directed phosphatase(s) may prove useful in modifying tumor progression.

Previous results indicate that ErbB2 increases activity of c-src in mammary epithelium. The present studies suggest that this induction of c-src activity is mediated by increased activity of a src-directed kinase that removes an inhibitory phosphate at Y527. SHP2 appears to be a leading candidate for the phosphatase. In addition, the activation of c-src by ErbB2 appears to play a critical role in inducing a tumor phenotype. These results that strategies to modify src activity or the activity of the src-directed phosphatase(s) may prove useful in modifying tumor progression.

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APPENDICES

Reprint

Abstract

C-Src Activation by ErbB2 Leads to Attachment-Independent Growth of Human Breast Epithelial Cells

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Nontumorigenic human mammary epithelial cells (184.A1 line) were stably transfected with ErbB2 or with Ha-Ras. Transformation with ErbB2, but not ras, resulted in a 5-6 fold increase in c-src activity without affecting c-src content of cells. Similar activation of c-src by ErbB2 was also observed in other non-tumorigenic mammary epithelial cells, including the human line MCF10A and the mouse line NMuMG. Activation of c-src appeared to be dependent on active ErbB2 tyrosine kinase, as the ErbB2 inhibitor tyrphostin AG 825 blocked the induction of c-src kinase activity, as well as the ability of transformed cells to grow on soft agar, but not plastic. The src-selective inhibitor PP1 effectively reduced c-src activity, as well as growth of ErbB2-transformed cells on soft agar, but not on plastic. These results indicate that activation of c-src is a consequence of ErbB2 kinase activity in human breast cancer cells overexpressing ErbB2, and that increased activity of c-src may be responsible for attachment-independent growth of the cells. © 1998 Academic Press

ErbB2 (neu) is overexpressed in 10-30% of human breast cancers (1). ErbB2 expression has been associated with aggressively growing tumors and poor prognosis (2, 3). Such effects are thought to be a result of ErbB2 activity, rather than correlatory effect, since ErbB2 expression in rodent mammary tissue by either in situ gene transfer or transgenic technology results in the development of mammary carcinoma at young ages (4-7). Furthermore, nontumorigenic human mammary epithelial cells can be tumorigenically transformed by expression of ErbB2 (8, 9).

The product of the ErbB2 gene is a tyrosine kinase in the same family as EGF receptor, but with no known ligand (10). A variety of signal transduction events mediated by ErbB2 have been described, including activation of ras-MAPK and PI_3 Kinase pathways (11, 12). In mice transgenic for ErbB2, tumor tissue contains elevated c-src activity relative to non-tumor tissue (13).

These results suggest that ErbB2 overexpression causes an activation of c-src, which may be at least partly responsible for the phenotype of ErbB2 transformed cells. Therefore, the objective of this study was to determine if overexpressing ErbB2 results in activation of c-src in non-tumorigenic human mammary epithelial cells and if c-src activation may be responsible for tumor phenotype.

MATERIALS AND METHODS

Cell lines. 184.A1 human mammary epithelial cells (14), MCF10A human mammary epithelial cells (15) and NMuMG mouse mammary epithelial cells (16) were obtained from ATCC (Rockville, MD). Cells maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10 ng/ml EGF. pJRneu (17) and pJRras (18) were kindly provided by Dr. M. Gould (University of Wisconsin, Madison). pJRneu is based on the plasmid pJR containing tumorigenic human neu, driven by CMV promoter and containing a neomycin resistance marker. pJRras is similar, except that v-Ha-ras is present insead of neu. Transfected cell lines were selected in media containing 400 μg/ml G418 sulfate.

Western blot analysis. To assess expression of ErbB2 and c-src, cells were grown to near confluency and lysed with SDS loading buffer (19) lacking 2-mercaptoethanol and bromophenol blue. Protein content of samples was determined by BCA assay (Pierce Chemical Co., Rockford, IL), 2-mercaptoethanol and bromophenol blue added to the samples and 50 μg protein separated by SDS-PAGE. Proteins were transferred to PVDF membranes and western blots probed with antibodies against c-src (UBI, Lake Placid, NY) essentially as described by Fenton and Sheffield (20).

c-src activity. Cells grown to approximately 80% confluency were lysed with lysis buffer (50 mM HEPES, pH 7.0 containing 30 mM sodium pyrophosphate, 10 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100,m 0.1% BSA, 1 mM sodium orthovanadate and 1 mM PMSF) and centrifuged (15,000 g for 15 minutes). Protein content of the supernatant was determined by BCA assay, equalized among cell lines and c-src immunoprecipitated from equal protein amounts essentially as previously described (21) using anti-c-src (UBI) and agarose conjuated protein A and G (Santa Cruz Biotechnology, Santa Cruz, CA). Beads were washed 4 times with lysis buffer and c-src activity determined by incubating immunoprecipitated enzyme in 15 μl of assay buffer (200 mM HEPES, pH 7.0 containing 125 mM $MgCl_2$, 25 mM $MnCl_2$, and 0.25 mM sodium orthovanadate) with or without substrate peptide ([lys[19]]cdc2(6-20)) or control pep-

tide ([phe^{15}lys^{19}]cdc2(6-20)) (1 mM for standard assays). Reactions were started by adding 5 μM of a 0.5 mM γ^{32}P-ATP solution (approximately 1 Ci/mmole, Dupont, Boston, MA). Standard reactions were continued for 5 minutes and stopped by adding 10% trichloroacetic acid and 100 μg bovine serum albumin. Samples were centrifuged (3000 g for 5 minutes) and supernatant spotted onto Whattman P81 phosphocellulose paper. Paper was washed 5 times with 100 mM phosphoric acid, dried and counted by liquid scintillation. c-src content in immunoprecipitates was determined by western blot analysis as described above, quantitated by computer-assisted densitometry (Collage, Fotodyne, New Berlin, WI) and activity adjusted for c-src content.

Cell growth. Control or ErbB2 transfected cells were plated onto petri dishes (10^4 cells/cm^2), treated as described and allowed to grow for 3 days with media changed each day. Cells were counted by hemocytometer counting and population doubling time estimated as an index of growth rate.

Soft agar growth. Wild-type or ErbB2 transformed cells were suspended in DMEM containing 10% fetal bovine serum and 0.3 % agar .
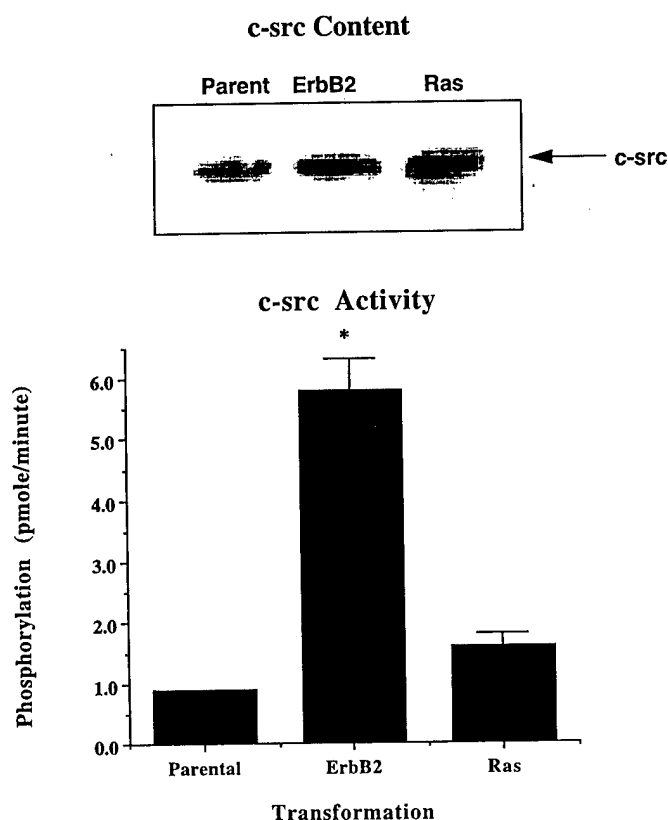
c-src Content

FIG. 1. c-src content and activity of parental, ErbB2 transformed and ras-transformed 184.A1 human mammary epithelial cells. For both studies, parental, ErbB2 and ras transformed cells were generated and cultured as described in Materials and Methods. A. Cells were lysed with SDS loading buffer, proteins separated by SDS PAGE and western blot analysis was performed as described in Materials and Methods. Representative of 3 experiments. Densitometry analysis indicated no significant differences in c-src expression. B. c-src was immunoprecipitated, c-src content of immunoprecipitates assessed by western analysis and kinase activity determined as described in Materials and Methods. c-src activity was reported prior to normalization to c-src content of immunoprecipitates. Mean ± SEM of 3 experiments. * = Significantly different than parental line, P<0.05.
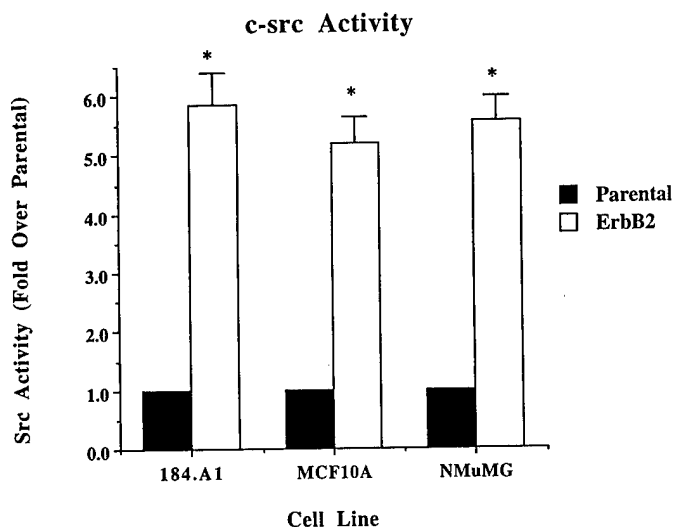
c-src Activity

FIG. 2. Comparison of cell lines. Parental, ErbB2 and ras transformed 184.A1, MCF-10A and NMuMG cells were generated as described in Materials and Methods. c-src was immunoprecipitated, c-src content of immunoprecipitates determined by western blot analysis and kinase activity of immunoprecipitates determined as described in Materials and Methods. Kinase activity was reported after normalization to c-src content of immunoprecipitates. Mean ± SEM of 3 determinations. * = Significantly different than parental line, P<0.05.

and plated over 0.5 % bottom agar at a density of 10^3 cells/100 mm petri dish. Cells were then treated as described and colony forming efficiency determined after 7 days of growth.

Statistical analysis. All experiments were replicated on at least 3 occasions. Quantitative data were analyzed by analysis of variance (ANOVA) and transformed cell lines compared with parental cells by Dunnett's t-test (22). Unless otherwise states, all differences noted were P<0.05.

RESULTS

The c-src content of parental, ErbB2 transformed and ras transformed cells did not vary significantly among the 3 lines (Figure 1A). Despite similar levels of c-src, phosphorylation of a c-src substrate peptide was increased approximately 5-6 fold in ErbB2 expressing cells compared with control cells (Figure 1B). The c-src activity in ras-transformed cells was not significantly different than parental cells, suggesting that this effect was not a general effect of transformation but was likely due to ErbB2 expression. In preliminary studies (not shown), c-src kinase activity was found to be dependent on the presence of substrate peptide, linear over time and linear over the amount of cell extract used for immunoprecipitation.

The effect of ErbB2 transformation on c-src activity was not confined to 184.A1 cells. The nontumorigenic human mammary epithelial cell line MCF-10A and the mouse line NMuMG also exhibited substantial increases in c-src activity upon transfection with ErbB2, but not with ras (Figure 2).
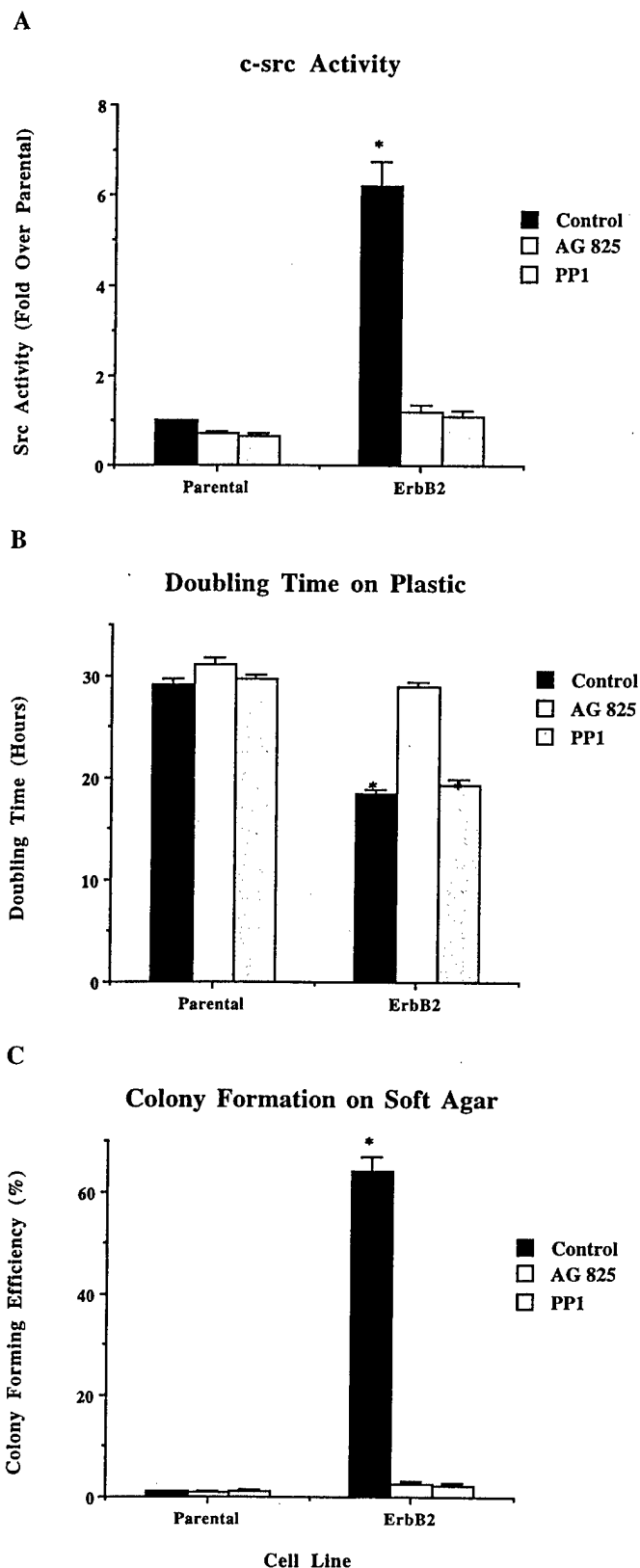
A

c-src Activity

B

Doubling Time on Plastic

C

Colony Formation on Soft Agar

Cell Line

FIG. 3. Influence of Tyrphostin AG 825 (ErbB2 inhibitor) or PP1 (src inhibitor) on c-src activity (A), growth on plastic (B) and growth on soft agar (C). Parental and ErbB2 transformed 184.A1 cells were generated as described in Materials and Methods, cultured on plastic

In order to further verify that the observed change in c-src activity was due to ErbB2 activity, the effects of the selective ErbB2 inhibitor Tyrphostin AG 825 on c-src activation, cell growth and attachment independent growth was assessed. AG 825 (10 μM) dramatically decreased c-src activity in ErbB2 transformed cells, to levels approximately equal to control levels (Figure 3A). This concentration had no effect on cell viability, as measured by trypan blue exclusion (not shown). Cell proliferation on plastic was slightly reduced by AG 825. Untreated ErbB2 transformed cells grew significantly faster than parental cells. However, AG 825 reduced the growth rate of ErbB2 transformed cells to rates that were not different than parental cells (Figure 3B). Parental cells were largely incapable of forming colonies on soft agar, while ErbB2 transformed cells readily formed colonies on soft agar. AG 825 dramatically inhibited the ability of ErbB2 transformed cells to form colonies on soft agar (Figure 3C).

In order to evaluate possible roles of c-src in the ErbB2 tumor phenotype, the ability of the putative c-src inhibitor PP1 to reverse tumor phenotype was examined. PP1 reduced c-src activity in ErbB2 transformed cells (Figure 3A). At a dose that resulted in c-src activity similar to control levels (100 nM), PP1 had little effect on cell proliferation on plastic and ErbB2 transformed cells continued to grow faster than parental cells (Figure 3B). However, PP1 dramatically inhibited growth of ErbB2 transformed cells on soft agar (Figure 3C). These results suggest that c-src activation by ErbB2 may have little effect on mitogenesis of human breast cancer, but may play an important role in other tumor phenotypes, such as attachment independent growth.

DISCUSSION

The present study indicates that overexpression of ErbB2 in a non-tumorigenic human mammary epithelial cell line, which has previously been shown to result in tumorigenic transformation of the cells (9), results in dramatically increased c-src activity, in the absence of altered c-src content. These results indicate that previously observed associations between ErbB2 expression and c-src activity (23-25) are likely to be a result of ErbB2 expression, and not a coincidental correlation.

(A and B) or soft agar (C) and treated with Tyrphostin AG 825 (10 μM, AG) or PP1 (100 nM). A. c-src was immunoprecipitated, c-src content determined by western blot analysis, kinase activity determined as described in Materials and Methods and kinase activity normalized to c-src content of immunoprecipitates. B. Cells were plated, cultured for 3 days and growth rate was expressed as population doubling time. C. Cells were plated onto soft agar, cultured for 7 days and colony forming efficiency was estimated. Mean ± SEM of 4 experiments. * = Significantly different than control treated parental cells, $P<0.05$.

Furthermore, the results indicate that previously reported increases in c-src activity in transgenic mice expressing ErbB2 in mammary tissue (13) is also observed in human mammary epithelium. The lack of effect of ras transformation suggests that the observed effect is not a general result of transfection, selection or transformation, but is likely due to specific signaling pathways.

Because ErbB2 is known to function as a tyrosine kinase (10), we also examined the effects of the ErbB2 selective kinase inhibitor Tyrphostin AG 825 on c-src activity. This inhibitor appears to have substantial selectivity for ErbB2 over other tyrosine kinases, including c-src and ErbB1 (26). However, as with all inhibitor studies, possible non-specific effects cannot be completely eliminated. Nonetheless, these studies suggest ErbB2-mediated c-src activation is likely to depend on the tyrosine kinase activity of ErbB2. However, the exact pathway involved is not elucidated in these studies. Presumably, the pathway could involve activation of phosphotyrosine phosphatases (9, 27), inhibition of CSK-like activities (28) or direct association with ErbB2 (29).

Functionally, c-src activation could mediate a variety of tumorous phenotypes. Guy et al. (30) observed that c-src ablation decreased Polyoma virus middle-T antigen-induced mammary tumorigenesis in transgenic mice. Other researchers have reported that c-src mediated signaling may be important in cell cycle progression (31). In addition, src is well known as a mediator of cytoskeletal architecture, cell adhesion and motility (32, 33). Such processes are clearly implicated in tumor metastasis (34). Members of the c-src family have also been associated with integrin signaling complexes, which may be involved in tumor development and metastasis (35). Since ErbB2 is associated with increased tumor invasiveness and poor prognosis, the hypothesis that c-src activation by ErbB2 contributes to the highly invasive phenotype of ErbB2 expressing tumors is attractive, but as yet unproven.

In order to evaluate the possible role of c-src in tumor development, the src family selective inhibitor PP1 was used. Originally, PP1 was described as an inhibitor of Lck and Fyn (36), but is also capable of inhibiting c-src (37). In contrast to recent studies on ErbB2-induced mouse mammary tumors, which found that PP1 did not affect attachment independent cell growth (38), the present study found that proliferation of ErbB2-transformed human mammary epithelium on plastic was unaffected by PP1, but soft agar growth was dramatically inhibited by PP1. These results suggest that c-src activation by ErbB2 may not be important in mediating cell cycle progression, but may be critical for mediating other aspects of the transformed phenotype.

ACKNOWLEDGMENTS

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INVOLVEMENT OF SHP2 IN ErbB2-MEDIATED TUMORIGENESIS

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Mammary epithelial cells were readily transformed by overexpression of ErbB2. Such transformed cells exhibit classical transformed phenotype, including attachment-independent growth and formation of tumors in athymic mice. ErbB2 transformation was associated with increased activity, but not expression, of c-src. Phosphopeptide mapping indicated that ErbB2-associated c-src exhibited greater phosphorylation at tyrosine 417 and lower phosphorylation at tyrosine 527. The dephosphorylation of tyrosine 527 was not associated with decreased amount or activity of C-Terminal Src Kinase (CSK), but was associated with increased activity of phosphotyrosine phosphatases directed toward the C-terminus of c-src. The phosphotyrosine phosphatase SHP2 was identified as a major phosphatase associated with the ErbB2-src signaling complex. Overexpression of an inactive SHP2 dramatically reduced the ability of ErbB2 to activate c-src. Furthermore, co-expression of ErbB2 and either kinase inactive src or inactive SHP2 inhibited various aspects of cell physiology stimulated by ErbB2. ErbB2 induced increased activity of focal adhesion kinase (FAK), which was decreased by both inactive src and SHP2. Similarly, ErbB2 induced increased tyrosine phosphorylation of several STAT proteins, including 1, 3 and 5. The tyrosine phosphorylation of STAT 3 and 5 was largely eliminated by overexpression of inactive src or SHP2. In addition, the ability of ErbB2 transformed cells to form tumors in athymic mice was reduced (but not eliminated) by overexpression of inactive src or SHP2. These results indicate that ErbB2 induces activation of c-src via the phosphotyrosine phosphatase SHP2, and that this activation may be required for some of the phenotype exhibited by ErbB2-transformed cells.